TITLE:

Pax3 and Pax7 play essential safeguard functions against environmental stress-induced birth defects

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SUMMARY:

Exposure to environmental teratogenic pollutant leads to severe birth defects. However, the biological events underlying these developmental abnormalities remain undefined. Here we report a molecular link between an environmental stress response pathway and key developmental genes during craniofacial development. Strikingly, mutant mice with impaired *Pax3/7* function display severe craniofacial defects. We show these are associated with an up-regulation of the signaling pathway mediated by the Aryl hydrocarbon Receptor (AHhR), the receptor to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), revealing a genetic interaction between *Pax3* and AhR AHR signaling. Activation of AhR AHR signaling in *Pax3*-deficient embryos drives facial mesenchymal cells out of the cell cycle through the up-regulation of *p21* expression. Accordingly, inhibiting AhR AHR activity rescues the cycling status of these cells and the facial closure of *Pax3/7* mutants. Together, our findings demonstrate that the regulation of AhR AHR signaling by Pax3/7 is required to protect against TCDD/AhRAHR-mediated teratogenesis during craniofacial development.

INTRODUCTION:

Exposure to environmental teratogenic pollutants is a major threat to embryonic development. In humans, among the population presenting birth defects, about 10-15% of these anomalies are due to exposure of pregnant women to environmental teratogenic pollutants (Gilbert-Barness, 2010). Among these developmental defects, craniofacial malformations represent a third of the defects observed (Dixon et al., 2011). It is postulated that most teratogens interfere with genetic programs regulating developmental processes (Dixon et al., 2011). Among environmental pollutants, TCDD commonly known as dioxin is a recognized potent teratogen (Yonemoto, 2000). During historical events such as the American Vietnam war or the Seveso incident, populations were exposed to high doses of TCDD (Mocarelli, 2001; Stellman et al., 2003). It was suggested that the spreading of TCDD led to an increase in congenital defects, however rigorous epidemiologic study establishing the link with TCDD exposure are lacking. Nevertheless, TCDD exposure has been associated with developmental alterations, including impaired psychomotor functions and cognition as well as reproductive and developmental defects (White and Birnbaum, 2009). Furthermore, mouse embryos exposed to TCDD present with hydronephrosis, thymic hypoplasia, craniofacial defects such as cleft palate, reduced weight and lethality at high doses (Courtney and Moore, 1971; King-Heiden et al., 2012; Kransler et al., 2007; Moore et al., 1973; Pratt et al., 1984; Yamada et al., 2006; Yonemoto, 2000).

~~AhR~~ AHR has been identified as the receptor to TCDD (Fernandez-Salguero et al., 1996; Hankinson, 1995), suggesting this signaling pathway could be involved in TCDD-mediated teratogenesis. While the etiology of TCDD-induced developmental defects remains unclear and studies linking TCDD exposure and oral cleft in human are lacking, palatal shelves from human,

rat and mouse display similar response to TCDD exposure *in vitro* (Abbott et al., 1999a), which suggests that human exposure to TCDD may be associated with oral clefts.

In vertebrates, the face mainly derives from the Neural Crest (NC), a transient structure that arises at the dorsal tip of the closing neural tube (Knecht and Bronner-Fraser, 2002). Cells from the neuroepithelium undergo an epithelial-mesenchymal transition prior to migrating to various regions of the embryo. Depending on their location along the anterior-posterior axis, these cells populate different structures and give rise to a large variety of cell types (Le Douarin et al., 2004). In the most rostral part of the embryo, Cranial Neural Crest Cells (CNCC) migrate ventro-laterally to colonize facial prominences (Birgbauer et al., 1995; Lumsden et al., 1991; Osumi-Yamashita et al., 1994; Serbedzija et al., 1992) where they participate in the formation of craniofacial bones, cartilage, connective tissue, neurons and glial cells (Couly et al., 1993; Dupin and Sommer, 2012; Knecht and Bronner-Fraser, 2002; Kontges and Lumsden, 1996; Noden and Trainor, 2005). Craniofacial malformations are generally linked to anomalies in CNCC development, part of them being due to teratogen exposure. Despite the identification of gene regulatory networks (GRNs) underpinning CNCC development (Betancur et al., 2010), little is known about how environmental pollutants interfere with these genetic networks during craniofacial development.

Central to these GRNs are genes coding for the paralogous paired-box transcription factors ~~Pax3~~ PAX3 and P~~ax~~AX7. These transcriptional regulators play a key role in the integration of inputs during NC induction and in controlling the specification of NC derivatives (Basch et al., 2006; Betancur et al., 2010; Minchin and Hughes, 2008; Monsoro-Burq et al., 2005; Sato et al., 2005). Although *Pax3* and *Pax7* function during early NC development is highly conserved among vertebrates (Betancur et al., 2010), their function during craniofacial formation is not understood.

Here we show that mutant mice with impaired *Pax3* and *Pax7* function display severe facial morphogenesis defects. Using a large-scale transcriptomic approach, we identified the ~~AhR~~ AHR signaling pathway as specifically up-regulated during craniofacial development of *Pax3/7* deficient mice, leading to precocious growth arrest in the cranial neural crest lineage. We further show that blocking ~~AhR~~ AHR signaling rescues facial growth and closure in mice with impaired *Pax3/7* function, while exposure to ~~AhR~~ AHR agonists such as TCDD leads to similar defects in embryos with reduced Pax3/7 activity, genetically linking *Pax3/7* with ~~*AhR*~~*Ahr*. Our data therefore demonstrate that important developmental genes play a key function to preserve the developing face from ~~AhR~~AHR/TCDD-mediated teratogenesis.

RESULTS:

Essential role of Pax3 and Pax7 during craniofacial development

Using mouse genetic models, we first examined the dynamics of *Pax3* and *Pax7* expression profiles during craniofacial development. As in other vertebrates (Nelms and Labosky, 2010), *Pax3* expression is detected within the anterior neural plate border prior to neural tube closure at embryonic day (E)8.5 (Figure S1A). ~~Pax3~~ PAX3 expression co-localizes with the NCC marker ~~Sox9~~ SOX9 (Nelms and Labosky, 2010; Figure S1B) and persists in several CNCC-derived tissues such as the frontonasal mass (FNM) and the medial and lateral nasal processes (MNP and LNP; Figure S1C). Of note, *Pax7* expression is restricted within *Pax3* domain; first observed in the FNM and the LNP, and later maintained in the LNP at E10.5 and E11.5 (Figure S1C and S1D).

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We next analyzed facial morphology in two distinct models with altered ~~Pax3~~ PAX3 and ~~Pax7~~ PAX7 functions (Figure 1A and 1B). While *Pax3* is required for NC induction in the sacral, trunk and vagal regions (Li et al., 1999; Van Ho et al., 2011), cranial NC induction occurs in our genetic models. However, a strong frontonasal dysplasia phenotype with a fully penetrant frontal cleft was observed in both $Pax3^{GFP/GFP}$; $Pax7^{LacZ/LacZ}$ double mutant and $Pax3^{Pax3\text{-}ERD/GFP}$ mutant embryos (Figures 1A, 1B and S2A). In the latter, cells in the *Pax3* positive lineage expressed a potent dominant negative form of Pax3 (*Pax3-ERD*) which alters both ~~Pax3~~ PAX3 and ~~Pax7~~ PAX7 function (Bajard et al., 2006). This phenotype contrasted with the medial fusion of the nasal processes observed in wild-type embryos and other compound *Pax3*/*Pax7* mutants (Figure S2B), supporting the notion that both proteins are required for facial development in mice. In *Pax3; Pax7* double mutant embryos, the phenotype was more severe than in $Pax3^{Pax3\text{-}ERD/GFP}$ embryos where the medial and lateral nasal swellings were rudimentary and failed to fuse

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resulting to a frontonasal cleft, whereas the maxillary and mandibular prominences were less affected (Figures 1A, 1B and S2A). Histological analyses at E11.5 and E13.5 revealed that the nasal process of both genetic models was severely diminished and the nasal septum bifurcated (Figure 1A and 1B).

Since CNCC reached the facial prominences and early specification is not affected in our genetic models (Figures 1C and 2A-C), we next assessed whether craniofacial defects observed in *Pax3*$^{Pax3\text{-}ERD/GFP}$ and *Pax3; Pax7* double mutant embryos were due to increased cell death of CNCC. Strikingly, we observed an increased proportion of apoptotic CNCC when both *Pax3* and *Pax7* were missing (Figure 2D). Furthermore, we were unable to detect any cell co-expressing GFP (~~Pax3~~PAX3) and β-galactosidase (~~Pax7~~PAX7) at E10.5 or E11.5 (data not shown), suggesting that the P~~ax~~AX3^{+}/~~Pax7~~PAX7^{+} cell population is lost in double mutant embryos, precluding further analysis in this genetic model.

In contrast, cell death was not increased in the CNCC of *Pax3*$^{Pax3\text{-}ERD/GFP}$ embryos (Figure 2D). Thus, we tested whether the frontal cleft face phenotype observed in *Pax3*$^{Pax3\text{-}ERD/GFP}$ could be a consequence of tissue misspecification. We observed impaired expression of *Msx1* in the MNP and LNP (Figure 2E) associated with reduced expression of *Pax7* and *Pax9* in the LNP (Figure S3A), suggesting a proliferation defect in the nasal processes of *Pax3*$^{Pax3\text{-}ERD/GFP}$ embryos (Bhatt et al., 2013; Houzelstein et al., 1997; Nelms and Labosky, 2010). In addition, *Dlx2* expression in nasal pits was barely detectable (Figure 2E) implying patterning defects of the nasal process in *Pax3*$^{Pax3\text{-}ERD/GFP}$ embryos (McKeown et al., 2005; Thomas et al., 2000). As confirmed by skeletal staining at E17.5, *Pax3*$^{Pax3\text{-}ERD/GFP}$ fetuses primarily presented with defects in the premaxilla and the palate bones (Figure S3B). The primary palate was almost completely absent, while the

secondary palate was formed but divided in two halves in *Pax3*$^{Pax3\text{-}ERD/GFP}$ embryos (Figures 2F and S3B).

Collectively, these data demonstrate that *Pax3* and *Pax7* are essential in regulating morphogenesis, survival, patterning and specification of the frontonasal structures during facial development.

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Up-regulation of ~~AhR~~ AHR signaling pathway expression is associated with impaired Pax3/7 function

In order to gain insight into the molecular mechanism by which *Pax3* and *Pax7* regulate craniofacial development, we performed a microarray screen to compare the transcriptomes of the facial prominences of *Pax3*$^{Pax3\text{-}ERD/GFP}$ mutant and *Pax3*$^{GFP/+}$ embryos at E11.5 (Figure 3A). We identified 76 up-regulated (Table S1) and 44 down-regulated (Table S2) genes in cells with impaired *Pax3/7* function compared to the control (Figure 3A). Among the deregulated genes, the most highly represented were components of the ~~AhR~~ AHR signaling pathway that were up-regulated in our screen, suggesting a strong activation of this signaling pathway in *Pax3*$^{Pax3\text{-}ERD/GFP}$ embryos.

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Transcripts levels for ~~*AhR*~~ *Ahr* and its direct target genes, including *Aldh1a3*, a gene coding for an enzyme involved in retinoic acid production and nasal process development (Dupe et al., 2003; Hankinson, 1995) and *p21* (*Cdkn1a*, cyclin-dependent kinase inhibitor 1A), a mediator of cell cycle exit and growth arrest (Barnes-Ellerbe et al., 2004; Besson et al., 2008), were higher in *Pax3*$^{Pax3\text{-}ERD/GFP}$ CNCC (Figure 3B). Consistently, we detected a specific up-regulation of ~~AhR~~ AHR protein in the nasal process mesenchyme and epithelium in both E10.5 and E11.5 *Pax3*$^{Pax3\text{-}ERD/GFP}$ embryos compare to control (Figures 3C and S4). *In situ* hybridization for *Aldh1a3* transcripts revealed that its expression in the nasal process was shifted to more anterior and

medial positions within this tissue (Figure 3D), reinforcing the notion that ~~AhR~~ AHR signaling activity domain is increased when the function of P~~ax~~AX3/7 is impaired. Furthermore, in the frontonasal ectoderm, *Aldh1a3* was previously shown to regulate *Fgf8* expression, a mediator of CNCC growth in the underlying mesenchyme (Dupe et al., 2003; Hu et al., 2003). Consistent with this, expression of *Fgf8* was lost in the nasal epithelium of $Pax3^{Pax3\text{-}ERD/GFP}$ embryos (Figure 3D), suggesting a decreased proliferation of the underlying mesenchymal CNCC that could explain the smaller nasal prominence phenotype. In addition, CNCC isolated from facial prominences exposed to TCDD, specifically induced *Aldh1a3* and *p21* expression (Figure 3E) demonstrating the responsiveness of ~~AhR~~ AHR signaling to environmental pollutants in this tissue.

Impaired Pax3/7 function leads to CNCC growth arrest and frontal cleft face

Migration of the CNCC takes place in $Pax3^{Pax3\text{-}ERD/GFP}$ embryos (Figure 1C), since the number of GFP^+ cells was essentially the same in $Pax3^{Pax3\text{-}ERD/GFP}$ and control embryos at E9.5 (Figure 4A). However from E10.5 onwards the number of GFP^+ cells within the frontonasal region was decreased by 30% in $Pax3^{Pax3\text{-}ERD/GFP}$ embryos compared to control (Figure 4A). Moreover, by E11.5 the proportion of GFP^+ FACS-sorted cells over the total number of cells was severely diminished by 34%, in $Pax3^{Pax3\text{-}ERD/GFP}$ embryos compared to the control (Figure 4B and 4C). This decrease in the ~~Pax3~~PAX3$^+$/~~Pax7~~PAX7$^+$ population correlated with a decline in the number of cycling cells in this population (Figure 4D and 4F) which explains the overall reduction of nasal process size, leading to a frontal cleft face.

As we found a marked up-regulation of the growth arrest gene *p21* in $Pax3^{Pax3\text{-}ERD/GFP}$ embryos, we hypothesized that *p21* induction might lead to growth failure of the nasal process, resulting in the frontal cleft face phenotype. Immunostaining for p21 and ~~Pax7~~ PAX7 demonstrated that 43%

of the cells had exited the cell cycle in *Pax3*$^{Pax3\text{-}ERD/GFP}$ embryos, while only 26% of the cells normally did so at E10.5 (Figure 4E and 4G). This increased cell cycle exit was also seen at E11.5 (Figure 4G). In addition, this growth arrest was associated with a decrease in the CNCC progenitor pool, as shown by analysis of AP2α and ~~Sox9~~ SOX9 expression (Figure 4H and 4I). Together these results suggest that during facial prominences growth, up-regulation of ~~AhR~~ AHR signaling pathway in CNCC drives these cells out of the cell cycle. Hence, this precocious cell cycle exit generates reduced craniofacial prominences unable to fuse together, resulting in the formation of a frontal cleft face.

Pax3/7-mediated regulation of ~~AhR~~ AHR signaling allows CNCC growth during craniofacial development

In order to demonstrate that up-regulation of ~~AhR~~ AHR signaling leads to the morphological defects observed in *Pax3*$^{Pax3\text{-}ERD/GFP}$ embryos, we treated control and mutant embryos with α-naphthoflavone, an ~~AhR~~ AHR antagonist (Jang et al., 2007). Treatments were performed by daily gavages administrated from E8.5 to E11.5. Strikingly, a considerable proportion (39%) of treated *Pax3*$^{Pax3\text{-}ERD/GFP}$ embryos exhibited a rescue of the frontal cleft face at E13.5 compared to the DMSO-treated *Pax3*$^{Pax3\text{-}ERD/GFP}$ embryos, which all display a frontal cleft face (Figure 5A and 5C). Histological analysis of the rescued embryos revealed that the nasal septum was fused in its medial part (Figure 5A). In addition, we observed that ~~AhR~~ AHR inhibition was sufficient to rescue primary palate formation (Figure 5B). Moreover, as the alignment of the prominent rugae suggested, the fusion of the secondary palate appeared to be rescued but delayed in time when compared with DMSO-treated *Pax3*$^{Pax3\text{-}ERD/GFP}$ embryos which present with a bifurcated secondary palate (Figure 5B).

Following inhibition of ~~AhR~~ AHR signaling with α-naphthoflavone, the number of cycling ~~Pax3~~PAX3^{+}/~~Pax7~~PAX7^{+} cells in both control and mutant embryos reached a similar level as in the DMSO-treated *Pax3*$^{GFP/+}$ control embryos (Figure 5D). Hence, inhibition of ~~AhR~~ AHR signaling in *Pax3*$^{Pax3\text{-}ERD/GFP}$ embryos was sufficient to rescue the proportion of proliferating cells in the ~~Pax3~~PAX3^{+}/~~Pax7~~PAX7^{+} population.

To demonstrate that the rescue of the number of cycling CNCC was due to the inhibition of ~~AhR~~ AHR signaling, we quantified the expression of *p21* in these embryos. As anticipated, in nearly half of α-naphthoflavone-treated *Pax3*$^{Pax3\text{-}ERD/GFP}$ embryos the expression level of *p21* within the facial prominences reached similar levels compared to that observed in DMSO-treated *Pax3*$^{GFP/+}$ control embryos (Figure 5E), demonstrating that the specific inhibition of ~~AhR~~ AHR signaling was sufficient to rescue the number of cycling CNCC in the facial prominences of these embryos.

We also tested β-naphthoflavone, an ~~AhR~~ AHR agonist (Swanson and Perdew, 1993). Strikingly, nearly all β-naphthoflavone-treated *Pax3*$^{GFP/GFP}$ or *Pax3*$^{Pax3\text{-}ERD/GFP}$ mutant embryos presented with an early lethality by E13.5. By contrast, *WT* and *Pax3*$^{GFP/+}$ embryos all developed normally (data not shown). The cause of death was not determined but this further demonstrates a strong genetic link between *Pax3*/*Pax7* and ~~*AhR*~~ *Ahr* signaling during development.

Altogether, our results demonstrate that during facial morphogenesis P~~ax~~AX3 and ~~Pax7~~ PAX7 act by regulating CNCC growth through the action of ~~AhR~~ AHR signaling.

Pax3 and Pax7 play a safeguard function against TCDD-induced craniofacial defects.

To further establish the link between *Pax3/7* function and ~~AhR~~ AHR signaling during craniofacial development, we speculated that they act by restricting the input of ~~AhR~~ AHR signaling to specific locations in the facial prominences. We therefore hypothesized that

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reducing ~~Pax3~~PAX3/7 activity in embryos exposed to TCDD should lead to facial defects. Accordingly, we used TCDD to stimulate ~~AhR~~ AHR signaling in a *Pax3*-null context. To this end, we intercrossed *Pax3*$^{GFP/+}$ mice and treated the pregnant females with a low dose of TCDD. As expected, *p21* expression was significantly up-regulated in both TCDD-treated *Pax3*$^{GFP/+}$ and *Pax3*$^{GFP/GFP}$ facial prominences (Figure 6A) confirming that ~~AhR~~ AHR signaling was activated. As expected, *WT* and *Pax3*$^{GFP/+}$ control embryos were normally formed. Yet, while all vehicle-treated control *Pax3*$^{GFP/GFP}$ mutant embryos displayed a normal craniofacial phenotype, 56% of the TCDD-treated *Pax3*$^{GFP/GFP}$ mutant embryos presented with a frontal cleft face phenotype (Figure 6B and 6C). This phenotype is reminiscent to the one observed in *Pax3*$^{Pax3\text{-}ERD/GFP}$ embryos (Figure 1B) and confirms that when ~~Pax3~~ PAX3 function is impaired, activating ~~AhR~~ AHR signaling is sufficient to generate craniofacial defects.

Collectively, our results reveal an unexpected safeguard function for ~~Pax3~~PAX3/7 during face morphogenesis whereby they restrict ~~AhR~~ AHR signaling input in order to allow the correct growth and maintenance of CNCC during craniofacial development.

DISCUSSION:

TCDD, and derived compounds such as Polychlorodibenzo-p-dioxines (PCDDs) and Polychlorodibenzofurane (PCDFs), are highly toxic persistent chemicals released into the environment as unintentional by-products of incomplete combustion of fossil fuels and wood, and during the incineration of municipal and industrial wastes. In humans, most of the exposure occurs through food, mainly meat and dairy products, fish and shellfish. The developing fetus is especially sensitive to TCDD exposure. It is therefore important to monitor the *in vivo* cellular response to TCDD during development in order to understand and prevent the molecular and cellular response following exposure to dioxins.

We show here that during morphogenesis of the face, ~~Pax3~~ PAX3 and ~~Pax7~~ PAX7 regulate the environmental stress response pathway mediated by ~~AhR~~ AHR signaling. Restriction of ~~AhR~~ AHR signaling by ~~Pax3~~ PAX3 and ~~Pax7~~ PAX7 highlights a key function for this pathway in controlling CNCC proliferation during craniofacial development.

Interestingly, inhibition of ~~AhR~~ AHR signaling only rescues the craniofacial phenotype of *Pax3*$^{Pax3\text{-}ERD/GFP}$ mutant embryos. Severe defects in peripheral nervous system (PNS) and muscle development are maintained in the trunk of these embryos (Bajard et al., 2006; Van Ho et al., 2011). This suggests that fundamental divergences exist between the GRNs controlling cranial and trunk neural crest development, possibly due to the late appearance of the head during evolution. Moreover, it points out that the regulation of ~~AhR~~ AHR signaling by *Pax3* may be a cranial neural crest-specific feature. In the trunk, *Pax3* has been shown to control migration and early specification of NCC generating the PNS (Tremblay et al., 1995; Van Ho et al., 2011). In the head, our data demonstrate that *Pax3* is mainly involved in regulating the cell cycle progression of CNCC via the regulation of ~~AhR~~ AHR signaling. This probably reflects the wider

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range of derivatives that CNCC normally generate compare to trunk NCC. Our study has been mainly focusing on mesenchymal cells however, analysis of cranial PNS development in *Pax3*$^{Pax3\text{-}ERD/GFP}$ mutant embryos would further confirm or refute the divergence between trunk and cranial GRNs governing NCC development.

In addition, the study of our mouse mutants for *Pax3* and *Pax7* revealed that one copy of either of these two genes is sufficient to allow correct craniofacial development, demonstrating a strong functional conservation (Figure S2B). Interestingly, in *Pax3*$^{Pax7/Pax7}$embryos, migration of neural crest cells is not affected (Relaix et al., 2004). This can suggest that *Pax3* and *Pax7* functions are notably redundant during CNCC development or, alternatively it can also reflect that GRNs controlling formation of the face are more robust than the ones controlling muscle development. The latter hypothesis seems more plausible when looking at the complexity of the GRNs controlling each different steps of CNCC development (Betancur et al., 2010), with several transcription factors being described to be binding to the same target gene to ensure its correct expression during the different processes of craniofacial development.

In chick and *Xenopus*, *Pax3* and *Pax7* have been described as essential factors for the induction and formation of the neural crest (Basch et al., 2006; Monsoro-Burq et al., 2005; Sato et al., 2005). However, in our mouse genetic models, despite the absence of *Pax3/7* or when their function is impaired, we observed normal NC induction and migration in the facial prominences of the embryo (Figure 1C). Whether this observation reflects a change in *Pax3/7* function during evolution and how they became associated with ~~AhR~~ AHR latter during development remains an open question. Our data reveal that unlike chick and Xenopus, mouse *Pax3/7* are not essential during early NC development but are involved in late craniofacial development to control the maintenance of a cycling CNCC population. It is plausible that the association with AhR

signaling, which only starts to be significantly expressed from E10.5 (Abbott et al., 1995; Jain et al., 1998), was co-opted during evolution as a mechanism to control cell cycle of CNCC derivatives. It is believed that ~~AhR~~ AHR protein evolved about 450 million years ago (Hankinson, 1995). Despite pre-existing natural sources of TCDD, a marked increase in environmental TCDD release occurred in the 19th century and still persist today due to anthropogenic causes (Weber et al., 2008). Thus, it is tempting to speculate that ~~AhR~~ AHR signaling has acquired different functions during evolution. The ability of ~~AhR~~ AHR to bind TCDD has made it a crucial regulator of pollution-induced teratogenesis.

In the facial prominences of the developing mouse embryo, *Ahr*~~R~~ expression is detected from E10.5 (Abbott et al., 1995; Jain et al., 1998), suggesting a potential role during early craniofacial development. Yet, ~~*AhR*~~*Ahr*-null mouse mutants that are non-sensitive to TCDD exposure (Fernandez-Salguero et al., 1996; Mimura et al., 1997), do not show craniofacial defects but present defects in liver and immune system development (Fernandez-Salguero et al., 1995). Indeed AhR is involved in the maintenance of hematopoeitic stem cells quiescence (Singh et al., 2011). Studies demonstrating the teratogenic effect of TCDD on mouse palate development (Abbott et al., 1994; Abbott et al., 1998; Abbott et al., 1999b; Courtney and Moore, 1971; Moore et al., 1973; Pratt et al., 1984; Yamada et al., 2006; Yonemoto, 2000) are consistent with our observation that ~~AhR~~ AHR signaling up-regulation is associated with the occurrence of craniofacial defects. During normal development, it was shown that ~~AhR~~ AHR is involved during secondary palate development and is controlled by the retinoic acid (RA) signaling pathway (Jacobs et al., 2011). Mutant mice missing components of the RA signaling down-regulate *Ah*~~*R*~~*r* expression and do not present with secondary palate clefts when exposed to TCDD (Jacobs et al., 2011). Additional studies performed in zebrafish reported that TCDD-

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induced craniofacial defects are mediated by AhR signaling. However, the defects are uniquely found in the lower jaw, affecting the structure of the mandible and the development of the Meckel's cartilage (Keller et al., 2008; Planchart and Mattingly, 2010) highlighting possible evolutionary divergences between zebrafish and mammals.

TCDD-mediated activation of ~~AhR~~ AHR signaling in a *Pax3*-null context is sufficient to trigger craniofacial defects, confirming a tight regulatory interaction between ~~Pax3~~PAX3/7 function and ~~AhR~~ AHR signaling to control growth of facial prominences in mouse. Accordingly, it was shown that ~~AhR~~ AHR directly binds the first intron of *p21 in vitro* (Barnes-Ellerbe et al., 2004; Dere et al., 2011; Lo and Matthews, 2012). Importantly, despite *p21* expression being up-regulated, all TCDD-treated *Pax3*$^{GFP/+}$ embryos developed normally, suggesting that additional GRNs downstream of Pax3 are also regulating craniofacial development. This will be investigated in future studies.

Altogether, our data show that during craniofacial development, *Pax3* and *Pax7* regulate ~~AhR~~ AHR signaling expression and activity. Restriction of ~~AhR~~ AHR signaling by *Pax3* and *Pax7* highlights a previously unknown key function for this pathway in controlling CNCC proliferation during craniofacial development.

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EXPERIMENTAL PROCEDURES:

Mutant mice for Pax3 and Pax7 and reagents

The mutant alleles for *Pax3* (*Pax3*^{nLacZ}, *Pax3*^{GFP} and *Pax3*$^{Pax3\text{-}ERD}$) and for *Pax7* (*Pax7*^{LacZ}) have been described previously (Bajard et al., 2006; Mansouri et al., 1996; Relaix et al., 2003; Relaix et al., 2005). Of note, the *Pax3*$^{Pax3\text{-}ERD}$ allele is a conditional one that drives the expression of the dominant negative form of Pax3 (Pax3-ERD) composed of the Pax3 DNA binding domain fused to the engrailed repressor domain (ERD) upon activation of a Cre recombinase. In this study, the Cre was driven by the zygote specific *PGK* enhancer (Lallemand et al., 1998). α-naphthoflavone and β-naphthoflavone (Sigma-Aldrich) were dissolved in DMSO then diluted in 1% carboxymethylcellulose (Sigma-Aldrich). α-naphthoflavone (7.5mg/kg/day) and β-naphthoflavone (30mg/kg/day) were then administered daily, orally to pregnant female mice from E8.5 until E11.5. TCDD dissolved in toluene (Sigma-Aldrich) was diluted in Corn Oil (Sigma-Aldrich). TCDD (4µg/kg/day) was then injected intra-peritoneally, to pregnant female mice from E8.5 until E11.5. All experiments were conducted according to the Institutional Animal Care and Use Committee of Paris 6 University.

Immunofluorescence and quantification

Mouse embryos from timed pregnant females were fixed by immersion in 4% paraformaldehyde/PBS for 45 min to 2 hours at 4°C. Fixed embryos were cryoprotected by equilibration in 30% sucrose/PBS, cryosectioned and processed for immunostaining as described in (Relaix et al., 2005). The following primary antibodies were used: mouse anti-~~AhR~~ AHR (Abcam, 1:100), rabbit anti-AP2α (Santa-Cruz, 1:200), rabbit anti-Cleaved-~~Caspase~~CASPASE~~-~~

3 (Cell Signaling, 1:100), mouse anti-~~Pax7~~ PAX7 (Developmental Studies Hybridoma Bank, 1:100), rabbit anti-Phospho-~~Histone~~HISTONE-3 (Millipore, 1:500), rabbit anti-p21 (ProteinTech, 1:50), and goat anti-~~Sox9~~ SOX9 (R&D Systems, 1:100). Secondary antibodies DyLight 649 donkey anti-mouse IgG (H+L), DyLight 649 donkey anti-rabbit IgG (H+L) and DyLight 649 goat anti-rabbit IgG (H+L) were purchased from Jackson Immuno Research and Alexa 594 goat anti-mouse IgG (H+L), Alexa 594 donkey anti-goat IgG (H+L) and Alexa 594 goat anti-rabbit IgG (H+L) from LifeTechnologies. Analysis was carried out using a Leica TCS SPE confocal microscope and images processed with Adobe Photoshop CS4 software (Adobe Systems). Cells were counted using ImageJ (version 1.46; National Institutes of Health, Bethesda, Maryland, USA) and Cell Counter plugin (Kurt De Vos, University of Sheffield, Academic Neurology) and were used to calculate the percentage of one cell population against another. Mean ± standard deviation was given. The single (*), double (**) and triple (***) asterisks represent P-values $P<0.05$, $P<0.005$ and $P<0.0001$ respectively by the Mann-Whitney non-parametric statistical test. All experiments have been performed on at least 3 independent embryos for each condition.

Histology, X-Gal and Dapi staining, skeletal preparation and mRNA in situ hybridization

For histology, sections of embryos prepared as for immunofluorescence were stained with Hematoxylin and Eosin (Vandenberg and Sassoon, 2009). X-Gal staining and whole mount *in situ* hybridization were performed as previously reported (Van Ho et al., 2011). For whole mount Dapi staining, protocol described by (Sandell et al., 2012) was used. Briefly, E15.5 embryos where fixed for 2 hours in 4% paraformaldehyde/PBS and washed 3 times with PBS before incubation with 6µg/ml Dapi in PBS overnight at 4°C. After 3 washes with PBS, embryos were

placed under a fully automatized Nikon Ti microscope and pictures were taken every 3µm in the z-axis to cover all the embryo thickness. The maximum projection of all the z-axis images using Fiji software revealed the fine topological details of the embryos. Staining of bones and cartilages of whole E17.5 embryos was performed as described in (Depew et al., 2002). For *in situ* hybridization, probes against the following mRNAs were used: *Dlx2* (Genbank Acc: BC094317), *Fgf8* (kindly provided by Martin G.), *Msx1* (Genbank Acc: BC016426), *Pax9* (Genbank Acc: BC005794). Analysis was carried out using a Leica MZ16 F stereomicroscope. Images were processed with Adobe Photoshop CS4 software (Adobe Systems). Analyses were performed on n≥3 embryos.

FACS sorting, cell culture and TCDD treatment

For FACS-sorting, CNCC were isolated from faces of E11.5 embryos initially incubated in digestion buffer [DMEM (LifeTechnologies), 0.1% Trypsin and 0.1% Collagenase D (Roche)] and purified via FACS Aria II based on gating of the GFP signal.

For TCDD treatment, CNCC were isolated from faces of E10.5 embryos, incubated in digestion buffer and purified using cell strainers (100µm then 40µm, BD Falcon) to obtain a single cell preparation. CNCC were then cultured in DMEM/F12 (1:1) medium for 24h with TCDD (100nM) or carrier in a collagen-plated dish.

RNA extraction, Microarray and RT-qPCR

RNA was extracted directly from dissected facial prominences or from FACS-sorted GFP^+ cells from facial prominences using NucleoSpin RNA II Extract kit (Macherey-Nagel) and the quality assessed with a Nanodrop ND-1000 (Thermo Scientific). cDNA was synthesized using the

Transcriptor First Strand cDNA Synthesis kit (Roche).

Microarrays were performed using Affimetrix GeneChip MOE130 2.0 (PartnerChip) chips containing 45000 oligonucleotide probes (25 mers) covering the totality of the 30000 genes of the mouse genome. Briefly, two-cycle cDNA synthesis was performed using 100ng of total RNA. cDNA was then hybridized to GeneChip Mouse Genome Array. Microarray analysis was performed using GeneChip Operating Software 1.4. For statistical analysis, data from three biological replicates of each genotype were averaged then normalized using the Affymetrix Mas5.0 algorithm. Statistical analysis was performed using BioConductor software (http://www.bioconductor.org/). Gene expression comparison between $Pax3^{Pax3\text{-}ERD/GFP}$ and $Pax3^{GFP/+}$ samples was performed using a statistical Student's t-test on normalized data. Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-3238.

RT-qPCR reactions were carried out in triplicate using the LightCycler 480 System (Roche). The expression of each gene was normalized to that of *Gapdh* transcripts. Results are given as mean ± standard deviation. The single (*), double (**) and triple (***) asterisks represent the P-values $P<0.05$, $P<0.005$ and $P<0.0001$ respectively for Student's unpaired t-tests. In Fig.4C, α–naphthoflavone treated $Pax3^{Pax3\text{-}ERD/GFP}$ embryos can be statistically segregated into two populations using a Ki^2 test. We compared the expression value for a given gene obtained in each α–naphthoflavone treated $Pax3^{Pax3\text{-}ERD/GFP}$ embryo to the mean of the expression values for all the DMSO treated $Pax3^{GFP/+}$ embryos (control set). If there were significant differences between these two values, the embryo was classified into the population described as responsive to the α–naphthoflavone treatment, if not the embryo was associated with the non-responsive population.

The following oligonucleotides were used:

~~*AhR*~~*Ahr*: (fwd) TTCCAGGTTCTCAGGCATTC; (rev) TGGGAGCTACAGGAATCCAC

Aldh1a3: (fwd) GCAGCAGTGTTCACCAAAAA; (rev) CCTCAGGGGTTCTTCTCCTC

p21: (fwd) GTACTTCCTCTGCCCTGCTG; (rev) GGGCACTTCAGGGTTTTCTC

Gapdh: (fwd) CATGTTCCAGTATGACTCCACTC; (rev) GGCCTCACCCCATTTGATGT

AUTHOR CONTRIBUTIONS:

AZ, designed and performed experiments, analyzed data and wrote the manuscript. RR, designed and performed experiments, analyzed data. FA and BC, designed and performed experiments. FR, oversaw the entire project, designed experiments, performed experiments, analyzed data and wrote the manuscript.

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FIGURE LEGENDS:

Figure 1. *Pax3* and *Pax7* are essential for facial development.

(A) Histological transverse sections across the head region of E11.5 *Pax3*$^{GFP/+}$*; Pax7*$^{LacZ/+}$, *Pax3*$^{GFP/GFP}$*; Pax7*$^{LacZ/LacZ}$ and *Pax3*$^{Pax3\text{-}ERD/GFP}$ embryos at two distinct levels of the nasal process, as indicated by black lines in the embryo scheme. H/E: hematoxylin eosin; LNP: lateral nasal process; MNP: medial nasal process; NP: nasal pit; NE: neuroepithelium; OE: olfactory epithelium; E: eye. Scale bar, 500µm. **(B)** Bright field and GFP expression (top and middle panels, facial views) of E13.5 embryos of *Pax3*$^{GFP/+}$*; Pax7*$^{LacZ/+}$, *Pax3*$^{GFP/GFP}$*; Pax7*$^{LacZ/LacZ}$ and *Pax3*$^{Pax3\text{-}ERD/GFP}$ embryos. Arrowheads represent normally formed nasal processes. White stars indicate rudimentary nasal processes. Bottom panels show histological transverse sections through the nasal processes of these embryos, as indicated by the black line in the embryo scheme. Black stars indicate divided nasal septum. H/E: hematoxylin eosin. Scale bars, 500µm. **(C)** GFP expression in E9.5 *Pax3*$^{GFP/+}$*; Pax7*$^{LacZ/+}$, *Pax3*$^{GFP/GFP}$*; Pax7*$^{LacZ/LacZ}$ and *Pax3*$^{Pax3\text{-}ERD/GFP}$ embryos (lateral views). Arrowheads indicate the CNCC migrating into the facial prominences. Scale bar, 200µm.

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Figure 2. *Pax3*$^{Pax3\text{-}ERD/GFP}$ embryos present a fully penetrant frontal cleft face phenotype.

(A) Immunostaining showing the expression of AP2α in red, ~~Pax7~~ PAX7 in white and GFP in green on transverse sections within the cranial regions of *Pax3*$^{GFP/+}$ and *Pax3*$^{Pax3\text{-}ERD/GFP}$ embryos at E9.5. Scale bar, 50µm. **(B-C)** Quantification of the proportion of AP2α^{+} (B) and ~~Sox9~~SOX9^{+} (C) cells within the ~~Pax7~~PAX7^{+} population found on transverse sections through the cranial regions of E9.5 *Pax*$^{GFP/+}$ and *Pax3*$^{Pax3\text{-}ERD/GFP}$ embryos. Error bars represent the standard deviation. **(D)** Percentage of Cleaved-~~Caspase3~~CASPASE-3^{+} cells in the ~~Pax3~~PAX3^{+} CNCC population of E11.5 embryos of the indicated genotype. Error bars represent the standard deviation. **(E)** Whole mount *in situ* hybridization for *Msx1* and *Dlx2* transcripts on E11.5 embryos of the indicated genotype. Arrowhead indicates *Dlx2* expression. Star shows its absence. Dotted lines indicate nasal pit location. LNP: lateral nasal process, MNP: medial nasal process, Mx: maxillary process, Md: mandibular process. Scale bars, 500µm. **(F)** Composite images of multiple fields showing the ~~O~~oral cavity of ~~Dapi~~ DAPI stained E15.5 embryos of the indicated genotype from which the lower jaw was removed. White arrowheads indicate the remaining of the primary palate. Black arrowheads indicate the bifurcated secondary palate. Mx: maxillary, pr: prominent rugae, pp: primary palate, sp: secondary palate, saf: site of apposition and fusion of palatal shelves, v: primordia of vibrissae. Scale bar, 500µm.

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Figure 3. Impaired *Pax3/7* function leads to ~~AhR~~ AHR signaling up-regulation.

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(A) Volcano plot showing the 44 down-regulated (DR) genes (green) and 76 up-regulated (UR) genes (red) in GFP^{+} cells FACS-isolated from dissected facial prominences of E11.5 *Pax3*$^{Pax3\text{-}ERD/GFP}$ compared to *Pax3*$^{GFP/+}$ control embryos. Blue dots indicate the position of ~~*AhR*~~*Ahr*, *Aldh1a3* and *p21* transcripts. **(B)** Relative expression of ~~*AhR*~~*Ahr*, *Aldhla3* and *p21* assayed by RT-qPCR in cells FACS-sorted for GFP from E11.5 embryos of the indicated genotype. Error bars represent the standard deviation. **(C)** Composite images of multiple fields showing ~~AhR~~ AHR expression in red and GFP in green on transverse sections within the cranial regions of E11.5 embryos of the indicated genotype. Scale bars, 50µm. OE: olfactory epithelium, LNP: lateral nasal process, MNP: medial nasal process. **(D)** Whole mount *in situ* hybridization for *Aldh1a3* and *Fgf8* transcripts on *Pax3*$^{Pax3\text{-}ERD/GFP}$ compared to *Pax3*$^{GFP/+}$ control embryos. Stars indicate increased area of transcripts expression for *Aldh1a3* or reduced expression for *Fgf8.* Scale bar, 500µm. **(E)** *Aldh1a3* and *p21* relative expression assayed by RT-qPCR in CNCC from E10.5 WT embryos, exposed to TCDD or carrier as indicated. Error bars represent the standard deviation.

Figure 4. Impairing *Pax3/7* function induces cell cycle exit of CNCC.

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(A) Quantification of the number of ~~Pax7~~PAX7$^+$ cells within the GFP$^+$ population on transverse sections of the cranial structures of E9.5 and E10.5 embryos of the indicated genotype **(B)** GFP expression in E11.5 embryos of the indicated genotypes (lateral views). Scale bar, 500µm. Red boxes delineate the facial prominences dissected to perform the FACS-sorting in C. **(C)** Percentage of FACS-sorted GFP$^+$ cells within the total population of cells from the dissected facial prominences of E11.5 embryos of the indicated genotype. Error bars represent the standard deviation. **(D-E)** Phospho-~~Histone~~ HISTONE-~~H~~3 (PH3) and p21 expression in red, ~~Pax7~~ PAX7 in white and GFP in green on transverse sections within the cranial regions of embryos at indicated genotypes and stages. Scale bars, 50µm. **(F, G, H, I)** Quantification of the proportion of PH3$^+$ (F), p21$^+$ (G) AP2α$^+$ (H) and ~~Sox9~~SOX9$^+$ (I) cells within the ~~Pax7~~PAX7$^+$ population in sections through the cranial regions of embryos with the stages and genotypes indicated. Error bars represent the standard deviation.

Figure 5. Genetic interaction between *Pax3* and ~~AhR~~ AHR signaling is essential for normal craniofacial development.

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(A) Bright field and GFP expression of E13.5 embryos of the indicated genotype treated with DMSO or α-naphthoflavone (facial views, top and middle panels). Arrowheads indicate divided nasal processes. Star marks its fusion. Bottom panels ~~show~~represent composite images of multiple fields showing ~~–~~histological transverse sections through the nasal processes of these embryos. Arrowheads indicate divided nasal septum. Star marks its fusion. Scale bars, 500µm. **(B)** Composite images of multiple fields showing the~~O~~ oral cavity of ~~Dapi~~ DAPI stained E15.5 embryos of the indicated genotype treated with DMSO or α-naphthoflavone from which the lower jaw was removed. White arrowheads indicate the remaining of the primary palate. Black arrowheads indicate the bifurcated secondary palate. Star marks the alignment of the rugae in the secondary palate of α-naphthoflavone-treated *Pax3*$^{Pax3\text{-}ERD/GFP}$ embryos. Mx: maxillary, pr: prominent rugae, pp: primary palate, sp: secondary palate, saf: site of apposition and fusion of palatal shelves, v: primordia of vibrissae. Scale bar, 500µm. **(C)** Number of untreated, DMSO- and α-naphthoflavone-treated *Pax3*$^{Pax3\text{-}ERD/GFP}$ embryos presenting with a frontal cleft face or a closed face with the stages indicated. At E11.5, for each embryo, classification of the α-naphthoflavone-treated *Pax3*$^{Pax3\text{-}ERD/GFP}$ embryos was based on the nasal process fusion (not occurring in non-rescued mutants) and the level of *p21* expression (up-regulated in non-rescued mutants compare to control embryos). **(D)** Percentage of PH3^{+} cells within the ~~Pax7~~PAX7^{+} CNCC population in E11.5 embryos of the indicated genotype treated with DMSO or α-naphthoflavone. Error bars represent the standard deviation. **(D)** *p21* relative expression assayed by RT-qPCR in dissected faces of independent E11.5 embryos of the indicated genotype treated

with DMSO or α-naphthoflavone. Dotted line represents *p21* expression level in DMSO control embryos.

Figure 6. ~~Pax3~~ PAX3 plays a safeguard function against TCDD-induced craniofacial defects.

(A) *p21* relative expression assayed by RT-qPCR in dissected faces of independent E11.5 embryos of the indicated genotype treated with carrier or TCDD. Dotted line represents *p21* expression level in carrier-treated control embryos. **(B)** Bright field and GFP expression of E13.5 embryos of the indicated genotype treated with carrier or TCDD (facial views, top and middle panels). Arrowheads indicate divided nasal processes. Scale bar, 500µm. **(C)** Number of carrier- and TCDD-treated E13.5 embryos presenting with a normal or a frontal cleft with the genotype indicated.